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PRINCIPAL INVESTIGATOR: Darlene A. Dartt, Ph.D.

CONTRACTING ORGANIZATION: Schepens Eye Research Institute
Boston, MA 02114

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Darlene A. Dartt, Ph.D.

Email: dartt@vision.eri.harvard.edu

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14. ABSTRACT

Successful completion of this project will advance the military's ability to develop effective visual displays in their sophisticated equipment; to treat retinas damaged by misuse of lasers; and treat battlefield injuries to the cornea and retina.

15. SUBJECT TERMS
None listed.

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Task 1: Russell Woods & Eliezer Peli

Task: To investigate the effect of scale changes, appearance modification, non-scene information and view depth on detection rates and dual task performance using augmented vision systems.

BODY

We completed analyses of the two-scene inattentional blindness studies (with overlaid ball and hand games), first testing the effect of cartooning on unexpected event detection and then testing the effect of attended scene on unexpected event detection. We prepared and submitted a paper, now in press, for the journal Ophthalmic and Physiological Optics, reporting the results. The on-line version of the paper contains samples of the video techniques used in the studies (Apfelbaum *et al.*, 2007). We reported that cartooning had no significant effect on the detection of unexpected events. While it did not mitigate inattentional blindness, it also did not materially impact the performance of the attended distractor task. Since we believe that cartooning is useful in augmented vision devices to aid in feature detection and in distinguishing the overlaid scenes, the lack of interference is encouraging. We also reported that attending to the scene that contained the unexpected events always resulted in their detection, even thought they were frequently undetected in identical overlaid presentations when the events were in the unattended scene. Thus it is attention, not any confusion related to the overlaying, that controls inattentional blindness. This had never been shown before.

Preparation of the study in which the same “shell-game” scene will be shown at two different scales, to investigate the effect of same vs. different contexts on inattentional blindness, has proved challenging and is ongoing. We have had several iterations of pilot videos, to determine the location, lighting, shell and table covering colors, video filtering and deinterlacing techniques, and timing of the game action and actions of observers and passersby. We tested many deinterlacing techniques and products before settling on the sophisticated HiCon software. That software performs dynamic motion compensation, which proved to be necessary in avoiding objectionable artifacts when deinterlacing cartooned video.

Preliminary tests with the videos have established that the distractor task of following the game action can be performed accurately enough to identify the shell containing the hidden “pea” if sufficient attention is paid, and that under those circumstances it is possible to miss the unexpected events visible in the wide view. We also established that we have met the objective of ensuring that the action can only be followed by paying attention to the close view of the performer’s hands, not the wide view. This was largely accomplished by finding shells whose color is different from that of the table covering but have the same luminance, so that their edges are not detected by the luminance-based edge filter. We are now preparing to test the detectability of the various unexpected events we have taped to identify those to be used in the full study.

We have just begun modifying the experiment control software from the first studies to meet the needs of the current study. We have completed development of a video player that accepts frames at a 60Hz rate that have a wide view in their upper half and a close view in the lower half, and alternates sending the halves at a 120Hz rate to the projector. We have also built a 3-button box for the subjects to use to indicate their choice of the pea location under one of the three shells.

Key Research Accomplishments:

- Results of the first two studies reported in a paper now in press.
- Those studies showed that cartooning does not affect inattentional blindness, but attention does.
- Complex video preparation for the study of the effect of context has been mastered.

Conclusions:

Significant progress as been made, but challenges have put us behind schedule. Funding of this effort is ending, but we expect to complete the current studies without further funding.

References:

Apfelbaum, H. L., Apfelbaum, D. H., Woods, R. L. and Peli, E. (2007) Inattentional blindness and augmented-vision displays: Effects of cartoon-like filtering and attended scene (In Press). *Ophthal. Physiol. Opt.*

Appendices:

OPO manuscript

TASK 2: Dong Feng Chen, Leader

Task: To rescue retinal neuron function, restore immune privilege, and promote wound healing after retinal laser burn with a Diode laser

INTRODUCTION:

The use of lasers is becoming increasingly widespread in medicine, research, industry, and notably, the military. Accordingly, a growing number of occupational eye accidents involve laser injuries, which are at present untreatable and the photoreceptor loss irreversible. Neuroregeneration and replacement approaches therefore hold a great promise for restoring vision in these patients.

Moreover, limiting the size of neuronal damage would also be imperative for the management of retinal injury after the laser burn. Although it has long been suspected that inflammation, glial scar formation, and wound healing responses contribute critically, the exact roles for these cellular processes in retinal laser injuries and recovery have not been well studied in great detail. In particular, the eye is a immune privilege site, a mechanism that protects the eye from unwarranted immune inflammation and glial reactions (1, 2). Although these responses are the natural conclusion of the wound healing process, they do lead to chronic damage to the eye and result in interference with vision. An emerging hypothesis has been that a combined strategy of neuroregenerative and replacement therapies and simultaneous limitation of the extent of neuroinflammation and the size of the scar is vital for effective treatment and management of retinal laser injury.

BODY:

Subtask 1: Promote neural transplantation therapy after laser retinal injury (Dr. Michael Young):

To investigate the integration, differentiation and connectivity of murine retinal progenitor cells (RPCs) transplanted into the subretinal space of mice with laser-induced damage to the outer retina, RPCs were harvested. Neural retinas of postnatal day 1 mice overexpressing enhanced green fluorescence protein (GFP) were dissociated and grown in neurobasal medium supplemented with epidermal growth factor (20 mg/ml). Photocoagulation was performed using a diode laser (spot size: 350 μm; power: 100 mW; duration: 100 ms; 10 spots per eye). Two μl of GFP+ donor cell suspension, containing ~2 x 10^5 cells were injected into the subretinal space. Mice were sacrificed by CO_2 inhalation 2 to 6 weeks after transplantation. Substantial numbers of cells had migrated and incorporated into the recipient neural retinal. Most of these integrated cells were correctly oriented within outer nuclear layer (ONL) around the retinal laser lesion, and had developed morphological features typical of mature photoreceptors. Some of the integrated cells in the ONL were double-labeled with GFP and photoreceptor-associated markers, including rhodopsin and recoverin, rom-1 and peripherin-2. Some of the integrated cells expressed the ribbon synapse protein bassoon and made synaptic contact with rod bipolar cells, identified by immunostaining with protein kinase C. These data indicate that transplanted RPCs could migrate, integrate and develop morphological features typical of mature photoreceptors in the laser injured retina.

Subtask 2: Stimulate neurogenesis and regeneration after retinal laser burn (Dr. Dong Feng Chen):

Retinal Müller glia in higher vertebrates have been reported to possess progenitor cell properties and the ability to generate new neurons after injury. To determine the signals that can activate this dormant capacity of Müller glia in adult mice, we studied their behavior under glutamate stimulation. Various concentrations of glutamate and its analogue alpha-aminoadipate, which specifically binds Müller glia, were injected subretinally in adult mice. Proliferating retinal cells were labeled by subretinal injection of 5'-bromo-2'-deoxyuridine (BrdU) followed by immunohistochemistry. Müller cell fates were analyzed in retinal sections using double immunolabeling with primary antibodies against Müller and other retinal specific cell markers. The effects of glutamate and alpha-aminoadipate were also determined in purified Müller cell cultures. While high levels of glutamate induces retinal damage, subtoxic levels of glutamate directly stimulates Müller glia to re-enter the cell cycle and induce neurogenesis in vivo and in purified Müller cell cultures. Alpha-aminoadipate that selectively targets glial cells also induced expression of progenitor cell markers by

Müller cells in vitro or stimulated Müller cell migration to the outer nuclear layer (ONL) and to differentiate into photoreceptors in vivo. Therefore, mature Müller glia in adult mice can be induced to dedifferentiate, migrate, and generate new retinal neurons and photoreceptor cells by alpha-aminoadipate or glutamate signaling. The results of this study suggest a novel potential strategy for treating retinal neurodegeneration, including retinitis pigmentosa and age-related macular degeneration, without transplanting exogenous cells.

Subtask 3: Restore immune privilege in mice with retinal laser burn injuries (Dr. Joan Stein-Streilein):

Previous funds from the DOD supported preliminary studies that examined the condition of immune privilege, glial changes and inflammatory responses after retinal laser burn to the back of the eye. We noted that retinal laser injury terminated ocular immune privilege (ACAID) and induced vigorous neuroinflammation and reactive gliosis in the burned eye. Interestingly, for the first time, our data revealed that retinal laser burn in the ipsilateral eye interfered with the ability of the contralateral eye to induce ACAID, suggesting that damage to the choroidal tissue and retinal neurons in one eye compromised the function of immune privilege in both eyes.

To begin to understand the mechanisms involved in the loss of immune privilege after RLB we evaluated changes in the cytokines that may have occurred in the eye. We chose to begin with IL-6 and TGF-β. IL-6 is a proinflammatory cytokine involved in many diseases including rheumatoid arthritis and uveitis (3). IL-6 in the presence of TGF-β could contribute to the loss of ACAID. More importantly, IL-6 and TGF-β influence naïve T-cells to become powerful inflammatory cytokine producing cells Th17(4-6). To test this, we analyzed the mRNA for IL-6 and TGFβ in eye extracts. In brief, C57BL/6 mice received RLB treatment (4 spots, 200 μm, 50 mW, 50ms) to the right eye. Both the burned (ipspilateral) and non-burned (contralateral) eyes were enucleated at 1hr, 6hr, and 8 hr post-RLB. mRNA was extracted from eye tissue (excluding lens) and subjected to RT-PCR. While TGF-β was present post-RLB in both the RLB and contralateral eyes (at 1, 6 and 8 hr); IL-6 was detected (6 and 8 hr post-RLB) only in the RLB eye.

We observed that mice that received antigen presenting cells (APC) from anterior chamber (a.c.) only group had a significant decrease in ear swelling compared to immunized mice (ACAID) but mice that received APC from RLB/a.c. donor mice showed no decrease in ear thickness compared to the positive control. Ear swelling is a measure of Delayed Type Hypersensitivity (DTH) reactions. Thus DTH was not suppressed and the APC did not transfer tolerance in RLB eyes.

Previous studies from our laboratory showed that the ACAID was lost in mice that received RLB in the right eye whether the antigen was introduced into the burned or non-burned eye. This is interesting to us, because there is no damage or inflammation in the left eye (non-burned). Also the blood ocular barrier is still intact in the left eye. Additionally, we found that RLB/a.c treatment to the right eye followed by OVA ear challenge, led to mice actually becoming immunized. Here we determined if a.c. inoculation to the contralateral eye also was immunizing. In this preliminary experiment the right eye received the RLB and the left eye received antigen (OVA) in the anterior chamber. Within a week experimental and control mice were challenged by inoculation of antigen into the ear pinnae. Surprisingly, we found that mice that received the above listed treatment were not immunized through the left eye. As expected, mice which received a.c., ear challenge did not have ear swelling.

To begin to understand the changes that occur after RLB we decided to compare the number of T-cells after RLB to naïve mice. To test this we performed RLB on C57BL/6 mice. Six days after RLB the spleen was removed, T cells were stained with anti-CD3 (CD3 is a marker for all T-cells) and subjected to flow cytometry. We found that RLB mice had an increased percentage of T-cells (54.44% of total spleen cell population) compared to naïve mice (39.07%). While, these data support the notion that T cells have proliferated into effector T cells. Control studies that compare the CD3+ of ACAID mice will shed additional light on this observation.

Subtask 4: Regulate retinal glial scarring in the mouse retina following diode laser photocoagulation (Dr. Bruce Ksander):

In the present study, we used immunochemistry and Western blots to examine expression of αB-crystallin following retinal laser burns. We used laser treated αB-crystallin knockout mice to determine if ααB-crystallin triggers either increased, or decreased gliosis and retinal scarring. The results of our experiments indicate that:

1. Alpha-B crystallin is expressed at high levels in the retinal cells that surround the laser burn site and also in the RPE cells. The increased expression of αB-crystallin coincided with the up regulation of GFAP, a marker of glial cells, and gliosis at the burn site.
2. When a retinal laser burn occurs in the absence of αB-crystallin, there was reduced glial scarring, indicating that αB-crystallin triggers *increased gliosis*.
3. However, a retinal laser burn in the absence of αB-crystallin also displayed slower healing of the blood / retinal barrier at the burn site.

Subtask 5: Regulate retinal inflammation and wound healing by controlling the responses of macrophages and macroglia in the laser burned retinas. (Drs. Sharmila Masli & Tat Fong Ng):

Microglia in the retina are considered resident macrophages. While these cells show distinct ramified morphology in their resting state, in response to various stimuli in the retina these cells are capable of changing their morphology to more amoeboid form. During this activation process these cells are known to migrate towards the stimulus and acquire the ability to phagocytose similar to macrophages. Activated microglia also alter expression of their cell surface markers similar to macrophages. Any form of injury in the retina can induce migration and activation of microglia to the site of injury. Such activated microglia phagocytose injured neurons or cells and clear the debris. Activated microglia are capable of secreting various factors that include neurotrophic factors like TGFβ, bFGF and thrombospondin (TSP) and neurotoxic factors like TNFα and nitric oxide. It is not clear if their migration to the site of injury is associated with wound healing and regeneration of damaged neurons or initiation of inflammation. Thrombospondin (TSP) is an extracellular matrix protein known to activate latent TGFβ and participate in the wound healing process. Immunosuppressive properties of TGFβ in the retinal microenvironment can prevent inflammation and further damage. We hypothesized that TSP plays an important role in recovery from retinal injury by promoting wound healing and supporting neuroprotective rather than inflammatory phenotype of microglia.

Determine microglial migration and activation and immune responses in the laser-treated retinas. Investigate the role of thronbospondin (TSP)1, a potent regulator of microglial function, in the migration and activation of microglia and the laser-induced retinal injury, using TSP1 knockout mice and/or mice receiving TSP injections.

We used TSP1 deficient mice (TSP1-/-) to examine response of microglia to laser-induced retinal injury. Control wild-type mice (C57BL/6) were used for the comparison. Eyes of these mice were irradiated with laser (350 □m, 150 mW and 150 ms per spot) to induce injury. Each retina was divided in 4 quadrants and each received 3 spots of laser exposure. Retina tissue was harvested at different times post-injury – day 1, 3, 14 and day 120. Tissue was processed to extract total RNA that was subjected to real-time PCR analysis to detect message levels for various costimulatory molecules and inflammatory cytokines expressed by activated microglia. Eyes enucleated at different times post-injury were cryo-protected and sectioned for immunohistochemistry to detect expression of markers associated with the activation of microglia. We noted that in uninjured TSP1-/- retina migrated microglia cells were detectable that expressed activation marker MHC class II supporting our hypothesis that TSP deficient microenvironment in the retina promotes activation and migration of microglia from their ganglion cell layer location. In response to laser injury such MHC class II positive cells were detected at the site of injury in both wild-type and TSP1-/- retina 1 day post-injury. However unlike the wild-type retina, in TSP1-/- retina such cells persisted until day 14. Histological examination of retina tissue in wild-type mice showed healing by day 14 post-injury but TSP1-/- retina showed significant damage up until 120 days post-injury. This damage was accompanied with aberrant migration of retinal epithelial cells indicating absence of healing. Compared to wild-type retina in TSP1-/- retina activated phenotype of microglia predominates as determined by detectable expression of co-stimulatory markers such as CD40 and CD86. Real-time PCR analysis of retina tissue demonstrated increased message levels for cytotoxic cytokine TNFα and co-stimulatory molecules in the absence of TSP.

Subtask 6: Determine the role of retinal pigmented epithelium (RPE) and hepatocyte growth factor (HGF) in a mouse model of retinal laser injury (Dr. Kameran Lashkari):

We have shown that laser injury disrupts the RPE monolayer resulting in aberrant migration of RPE cells into the outer retinal layer at the site of laser injury. Migration of RPE cells is associated with heightened wound response and may augment tissue damage caused by laser injury. In response to laser injury, the areas between the outer retinal layer and the choroid become disrupted. RPE cells are also observed migrating into the damaged outer retina. This migration is associated with wound healing responses of the RPE initiated by laser injury. Additionally, laser injury induces early apoptosis of the outer nuclear layer as early as 12 hours. The RT-PCR data indicate an upregulation of HGF and cMet/HGFR in response to laser injury. HGF message increased in retina to above 3 fold at 3 hours and slowly diminished until day 14. cMet/HGFR message increased in retina by over 2 fold by 12 hours and decreased after day 1. Surprisingly, the HGF and cMet/HGFR message in the eyecup diminished after laser treatment.

We have shown that cMet/HGFR is expressed in the RPE monolayer, as well as in the inner choroid, and retinal ganglion cell layer. In response to laser injury, the expression of cMet/HGFR is increased in the RPE monolayer and migrating RPE cells. Immunohistochemistry (IHC) of laser-treated retinal tissue indicates that cMet/HGFR receptor in indeed activated in the response to laser injury. Using phospho-specific antibodies against phospho-tyrosine sites on the receptor, cMet/HGFR is shown to be phosphorylated (activated) in the laser-treated RPE layer as well as in migrating RPE cells.

Using transgenic mouse system, we have examined the effects of laser injury in TPR-met mice. In TPR-met, the receptor is permanently dimerized (due to its fusion with TPR) and is constitutively activated. A significant amount of effort was spent to create mice that were TPR-met positive but negative for the RD (retinal degeneration gene). Our results show that TPR-met mice express a robust RPE layer and increased response to laser injury and RPE migration. We are currently examining the effects of cMet abrogation on laser injury and RPE migration using $cMet^{fl/fl}$ mice. In these mice a component of the genomic message (exon 16) of the receptor has been flanked by flox-p sites making it susceptible to splicing by Cre recombinase protein. These mice are being bred and will undergo laser injury as discussed above. Mice will be injected subretinally with a 9:1 combination of AAV-CRE/AAV-GFP virus which is expected to abrogate cMet/HGFR receptor activity. Successful viral delivery will be assessed visually by presence of green fluorscence around the area of injection. It is expected that these eyes will exhibit decreased RPE migration and destructive wound healing responses. Additionally, there will be no cMet/HGFR phosphorylation as the receptor has been genomically rendered inactive.

KEY RESEARCH ACCOMPLISHMENTS:

Subtask 1. Demonstrated that transplanted RPCs are capable of migrating, integrating, and developing morphological features typical of mature photoreceptors and establishing synapse contact with rod bipolar cells.

Subtask 2. Demonstrated that mature Müller glia in adult mice can be induced to dedifferentiate, migrate, and generate new retinal neurons and photoreceptor cells by alpha-aminoadipate or glutamate signaling. The results of this study suggest a novel potential strategy for treating retinal neurodegeneration, including retinitis pigmentosa and age-related macular degeneration, without transplanting exogenous cells.

Subtask 3. Demonstrated that IL-6 and TGF-β are both expressed in the laser injured retina of the ipsilateral eye, while only TGF-β is expressed in the contralateral eye, suggesting that the mechanisms underlying the loss of ACAID in both eyes are through two different pathways.

Subtask 4. Demonstrated that αB-crystallin has two apposing effects following a laser burn. Increased αB-crystallin in retinal cells coincides with increased gliosis, while increased αB-crystallin in RPE cells coincides with slower healing of the blood / retinal barrier.

Subtask 5. Demonstrated that, in the absence of TSP, laser injury of the retina results in exacerbated structural disruption of the tissue and that activated retinal microglia increase or prolong their expression of MHC class II and cytotoxic factor TNFα in the injured retina.

Subtask 6. Demonstrated that retinal laser injury upregulates HGF and cMet/HGF receptor, which may contribute to the constitutive activation or abrogation on wound healing responses, and designed short polypeptide inhibitors for possible clinical application.

REPORTABLE OUTCOMES:

Subtask 1. Three abstracts have been submitted. They include one to the Pacific Ocular Regenerative Biology Conference (Sept. 2007), Simches Research Syposium (Nov. 2007) and the Association for Research in Vision and Ophthalmology (April, 2008).

Subtask 2. One manuscript reporting the results has been accepted for publication by *Invest Ophthalmol Vis Sci.* (Appendix: manuscript attached).

Subtask 3. An abstract has been submitted to the 40th Annual Meeting of the Society for Leukocyte Biology. (Appendix; Abstract attached).

Subtask 4. A paper is in preparation reporting the results.

Subtask 5. A manuscript for publication is being prepared.

Subtask 6. The study will be presented in the next meeting of the Association for Research in Vision and Ophthalmology (ARVO). A manuscript is under preparation which includes the preliminary data submitted as part of the original manuscript.

CONCLUSIONS:

Mature Müller glia in adult mice possess retinal progenitor cell properties and can be induced to dedifferentiate, migrate, and generate new retinal neurons and photoreceptor cells by alpha-aminoadipate or glutamate signaling. Moreover, transplanted cells were also found to migrate and integrate into ONL of the retina with laser injury, differentiate into photoreceptors, and seem to form synaptic connections. These studies suggest that neuroregenerative and cell replacement therapy may be used as a treatment to restore vision for retinal laser injury.

Moreover, the loss of ACAID or retinal immune privilege and induction of neuroinflammation, reactive gliosis and microglial activation in the laser burned retina may be, in part, due to the influx of inflammatory cytokines and increased expression of αB-crystallin. Thus, following a retinal laser burn, the amount of gliosis and retinal scarring can be reduced by limiting the expression of the small heat shock protein αB-crystallin and restoration of TSP in the damaged retina. These therapeutic strategies may allow recovery from retinal injury.

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APPENDICES:

Publications

Takeda M, Takimiya A, Jiao J, Cho KS, Trevion SG, Matsuda T, and Chen DF. Alpha-aminoadipate induces Photoreceptor Regeneration and Progenitor Cell Properties of Muller Glia in Adult Mice. *Invest Ophthalmol Vis Sci.* 2008; *in press.*

Abstracts

Lucas KG, Qiao H, Stein-Streilein J. Retinal laser burn interferes with immune privilege of the eye Abstract 40th Annual Meeting of the Society for Leukocyte Biology, Cambridge, MA, 2007. appendix.

TASK 3: James Zieske, Leader

Task: To develop an anti-infective corneal bandage.

A. Subtasks

1. Subtask (1): Produce a self-assembled collagen gel comprising a natural, strong, clear collagenous matrix that is generated *de novo.*

2. Subtask (2): Investigate the bio-stability of the corneal bandage.

3. Subtask (3): Determine whether human umbilical cord mesenchymal stem cells (MSCs) can be differentiated into corneal endothelial cells.

4. Subtask (4): To determine the basis for S. aureus adherance to corneal epithelial cells.

B. Hypothesis

Currently, there is little that can be done to treat eye injuries on the battlefield. We propose to develop a corneal bandage consisting of naturally occurring matrix materials. Ideally, this bandage will adhere to the wound, provide protection, promote maintenance of the globe, and inhibit detrimental immune responses. We will use this corneal bandage to study wound healing and infection, toward the ultimate goal of designing a second-generation bandage that prevents infection. We envision this as a multi-step project, first attempting to create a temporary bandage and secondly attempting to generate a more permanent bandage that would be incorporated into the cornea.

C. Results

Subtask (1): Produce a self-assembled collagen gel comprising a natural, strong, clear collagenous matrix that is generated *de novo.* (Jeffrey Ruberti, PhD)

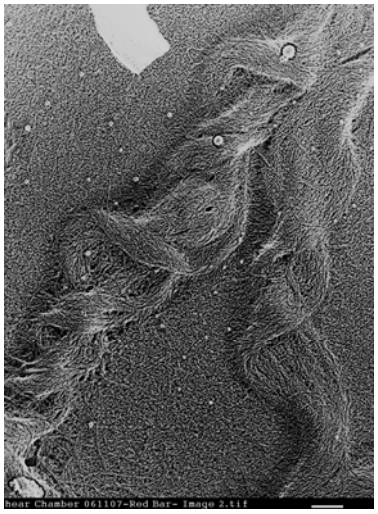
Dr. Ruberti's laboratory has been addressing two aims intended to produce an organized collagenous matrix, which can be used as a mechanically strong clear substrate into which cells and anti-inflammatory agents may be seeded. Our specific approach involves the development of two cell-free methods capable of controlling collagen assembly on the nanoscale:

1) **Influenced self-assembly in bulk thin shear film (Spin Coating)**
2) **Local control of collagen fibril assembly in nanoreactors (Collagen Nanoloom)**

Each of these approaches has the potential to generate aligned sheets of collagen fibrils similar to those found in the normal corneal stroma.

Progress in Year 2

Aim1: Influenced self-assembly in bulk thin shear film (Spin Coating)



A graduate student, Nima Saeidi, has been focusing on developing optimal parameters, which will allow shear and confinement to control the fibrillogenesis in thin films. In his work he has developed a shear chamber, which has been used to inform the work in the spin coating system. With the shear chamber, we are able to directly observe the polymerization of collagen on surfaces over which solution is flowing. From this work, we have found that the shear rate which produces aligned collagen can be as low as 7 sec^{-1}. Spin-coating of collagen produces even better alignment, but is very difficult to control (due to film instability). However, although shear-alignment produces reasonable alignment of fibrils, it was determined that the collagen fibril morphology was poor (**Figure 1**). Nima has been funded principally by the NIH, but some of the TATRC funds were used, as proposed, on this aspect of his work.

Figure 1. Quick-Freeze Deep Etch image of polymerized collagen on glass. The fibril has poor morphology (likely due to surface effects). Note clear resolution of the orientation of individual collagen monomers. Bar is 50 nm

Future work for Aim 1: Because of this difficult to address problem, the remainder of year 2 has focused on further development of the nanoloom.

Aim 2: Local control of collagen fibril assembly in nanoreactors (Collagen Nanoloom)

Nanoloom construction/design: During year 1, a device was constructed by Northeastern University undergraduates, which will allow us to test the hypothesis that collagen synthesis and organization can be controlled by simulating cell fibropositors. (The construction cost was funded by an NSF SGER award). Testing of the nanoloom has been carried through year 2 of the TATRC funding period by Katie Portale and Dr. Ericka Bueno. Katie has focused on nanoloom design and testing while Ericka has focused on transport of collagen.

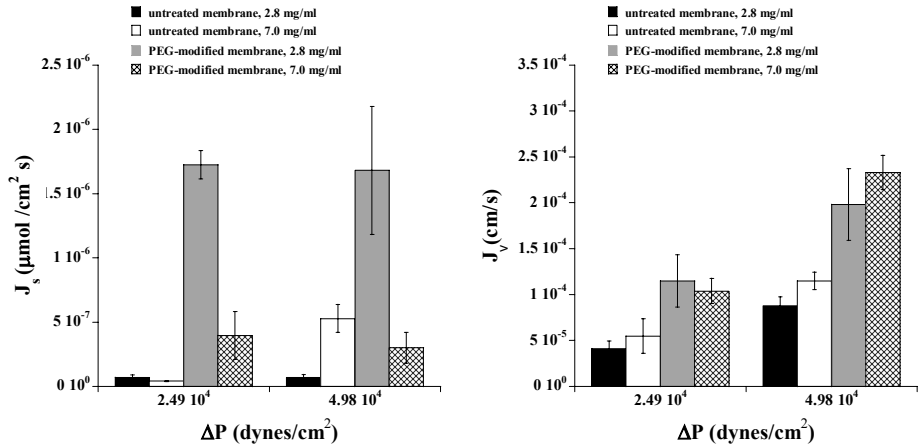


Figure 2. Flux of collagen (Left) and solution (Right) across polycarbonate track-etched membranes for various experimental conditions. There are two pressures (10 or 20 inH_2O), two concentrations (2.8 or 7.0 mg/ml) and two membrane surface conditions (PEGylated or untreated). The data demonstrate a potential maximum transport window at 10 inH_2O, 2.8 mg/ml using PEGylated polycarbonate membranes. (See next page for description of experiments)

Collagen transport. Dr Ericka Bueno has concluded a series of transport experiments designed to produce optimal parameters (pressure, concentration, membrane treatment) to elicit maximum collagen transport across the Nanoloom reactor arrays. The data indicate that collagen is a very complex macromolecule which will self-associate at nominal concentrations. Transport of solution and collagen was examined with a Ussing chamber designed to test flux across membranes (low-volume NaviCyte® single-channel vertical diffusion chamber - Harvard Apparatus, Holliston MA). The two graphs above summarize the results of the experiments which suggest that a window of maximum transport across the membrane occurs at 2.8 mg/ml and 10 inH_2O. Because the maximum porosity available in commercial track-etched membranes is relatively small (~2%), we are attempting to use newly developed (at NEU) aluminum oxide membranes which can be created with high aspect ratio pores and with nearly hexagonal close packing values of porosity. These membranes are also treatable. The results of this transport work have been compiled in a manuscript (near final draft attached) which will be submitted within two weeks of this progress report. The funding provided for this project has largely been applied to the work that was done by Dr. Bueno.

Figure 3. Collagen Nanoloom

Collagen fibrillogenesis in the Nanoloom. A graduate student, Kathryn Portale, has been investigating methods by which the polymerization of the collagen can be confined to one side of the track etched membrane (Nanoloom reactor array). By an accidental discovery, we found that collagen may be extruded in alignment into a bath with high ionic strength. Our explanation for this phenomenon is that the high strength salt solution causes the collagen monomers to collapse onto one another due to co-nonsolvency effects. This fortuitous discovery has led us to postulate that any solution which “steals” water from the collagen will make collapse. We have thus begun a series of experiments to test whether or not this is the case. The benefit of this effect is the fact that a steep temperature gradient may not be needed. Attempts were made to print collagen without fine temperature control, which encouraging results.

In figure 4, what we believe are our first few lines of printed collagen are shown. However, we have not proven that this is the case.

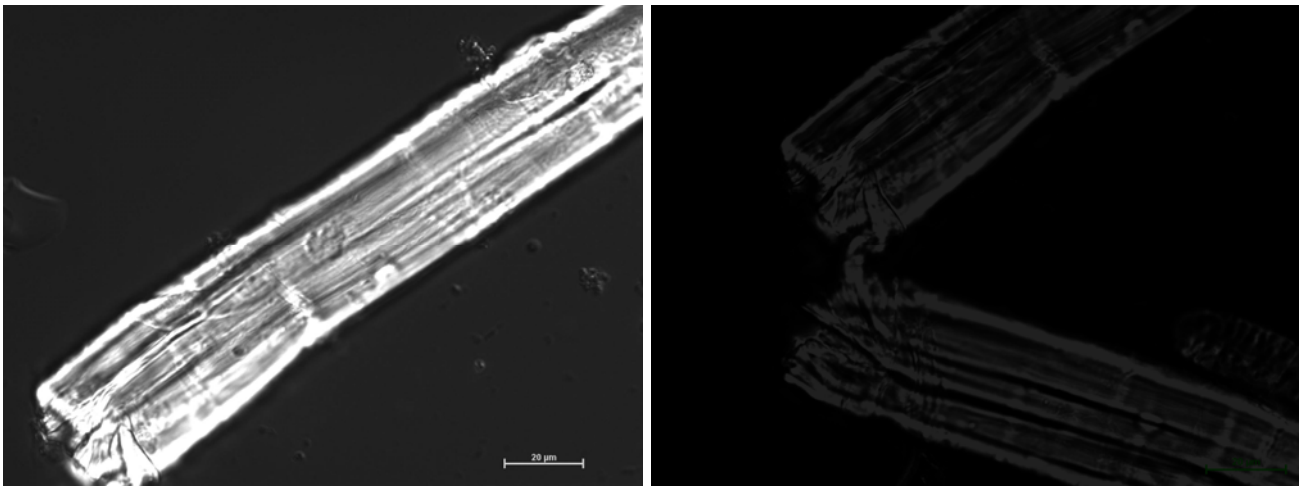


Figure 4. DIC images of strip of "collagen" printed by the nanoloom. The solution into which the collagen was printed was 10x PBS and scale bar is 30 microns.

Future work for Aim 2: Our intent is to continue to optimize the parameters which will make this device work. Changes include development of simple, directly observable test bed to enable dynamic views of collagen extrusion, use of high porosity alumina membranes, and printing into solutions with high osmotic pressures including polyethylene glycol (PEG) and hyaluronic acid.

Subtask (2): Investigate the bio-stability of the corneal bandage. (James Zieske, PhD)

Introduction: The goal of this subtask is to determine the response of the host to the corneal bandage by applying the bandage (artificial stroma) to mice using two experimental models. In the first model, a sheet of artificial stroma was implanted inside the recipient's stroma (intralamellar corneal transplantation model). In the second model, a sheet of the artificial stroma was placed on the surface of the recipient's stroma (onlay model).

Methods: Primary human fibroblasts were isolated from human donors, cultured, propagated in vitro, and characterized. The cells were seeded in Transwell inserts and then allowed to grow for four weeks. During this time the cells stratified and produced an extracellular matrix to form a 3-D construct. In the intralamellar model, cells were labeled with Q-tracker (fluorescent red) before transplant. In this model the construct was transplanted into BALB/c mice. The original protocol had called for the use of immunodeficient scid mice, but these proved to be unnecessary. To generate this model, a pouch was made in the stroma and the construct was placed in the pouch. Graft acceptance was indicated by the continuing presence of the Q-tracker fluorescence. Graft rejection was indicated by neovascularization and loss of fluorescence. In the onlay model, a 2-mm superficial keratectomy was made in the central cornea of BALB/c mice to expose the stroma. Rose Bengal (0.05%) was applied to the exposed corneal stromal surface, and the corneal bandage was laid down on the stroma and irradiated with a green laser (532nm) for 180 seconds. Prior to transplantation, the artificial stroma was labeled with DTAF. The attachment of the corneal bandage was examined by the presence of the DTAF. The mice were sacrificed and the eyes were processed and examined by immunofluorescence microscopy for the presence of infiltrating immune cells with antiCD45.

Results: The isolated corneal cells expressed keratan sulfate proteoglycan (KSPG) Ki67 and CD90. After forming the 3-D construct, the KSPG positive cells laid down extracellular matrix components including collagen VI, fibronectin, and thrombospondin 1. Pouch Model: In the intralamellar model, 75% of the constructs were maintained for 4 weeks and 50% were maintained for 8 weeks. The constructs did not appear to generate an immune response. Loss of fluorescence was accompanied by neovascularization. Onlay Model: In the onlay model, the Rose Bengal and the green laser irradiation made a tight bond

between the bandage and the corneal stroma. The DTAF labeled collagen stayed on the corneal surface for at least 14 days (one mouse maintained the bandage for 140 days). The construct was covered by epithelium by day 3. CD45 positive immune cells were found on day 1 in the limbus and central cornea in eyes +/- the artificial bandage. However, by day 3 the number of immune cells decreased in the corneas with the bandage; whereas, the corneas without bandage still maintained numerous CD45 positive cells.

Conclusions: The corneal bandage is accepted by the wounded recipient and provides a good barrier for protecting the injured cornea stroma.

Subtask (3): Determine whether human umbilical cord blood mesenchymal stem cells (MSCs) can be differentiated into corneal endothelial cells. (Nancy Joyce, Ph.D.)

INTRODUCTION: Corneal endothelium is the single layer of cells at the posterior of the cornea that is responsible for maintaining corneal clarity. Preparation of a clear, anti-infective corneal bandage therefore requires the presence of a functional layer of corneal endothelial cells. Although our laboratory has had consistent success in culturing human corneal endothelial cells (HCEC)[1-3], the proliferative capacity of these cells is limited and decreases with age[2-4]. Since the majority of donor corneas are obtained from older individuals, there is a limited ability of endothelial cells isolated from these corneas to be expanded in culture. Thus, the ability to develop a corneal bandage containing normal endothelium may be compromised. During eye development, corneal endothelium is formed from neural crest-derived mesenchymal cells that migrate beneath primary stromal material[5-7]. Because of this origin, HCEC share characteristics with neuronal cells, such as expression of neuron-specific enolase[7,8], S-100[7], and N-cadherin[9,10], while at the same time expressing the mesenchymal intermediate filament protein, vimentin[7]. Multipotent mesenchymal stem cells obtained from post-partum human umbilical cord blood have the capacity to differentiate into several cell types, including neurogenic cells[11,12]. As such, we **hypothesize** that human umbilical cord blood mesenchymal stem cells (MSCs) can be differentiated into HCEC to provide a ready supply of cells for tissue bioengineering. This is a NOVEL APPROACH, which could have a significant impact on the development of a clear, anti-infective corneal bandage, as well as on treatment of corneal blindness in patients with corneal endothelial dysfunction.

SPECIFIC AIM: The single Specific Aim of this sub-project is to determine whether human umbilical cord blood mesenchymal stem cells (MSCs) can be differentiated into corneal endothelial cells.

RESEARCH ACCOMPLISHMENTS: We obtained six clones of human umbilical cord blood MSCs from Dr. Biagio Saitta, Coriell Institute for Medical Research, Camden, NJ. Umbilical cord blood was obtained after full-term deliveries. Donor confidentiality was maintained in accordance with the requirements of the Internal Review Boards at Coriell and Schepens.

1) Expansion of MSC: All MSC cloned cultures were passaged under basal conditions (DMEM with low glucose, 10% FBS) to expand cell numbers. Cells were frozen in liquid nitrogen to maintain their stem-like characteristics. Two of these cultures (MSC 34b and MSC 44) were used to conduct initial tests. Images of these cultures are presented in Figure 1.

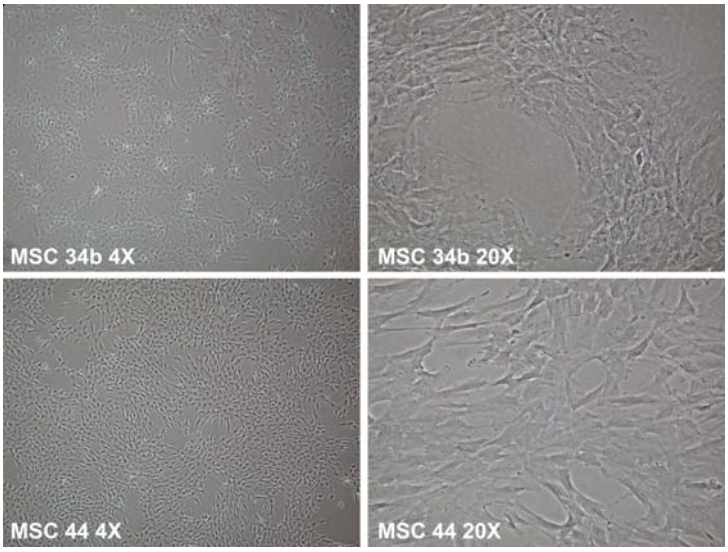


Figure 1. Two clones of human umbilical cord blood mesenchymal stem cells (MSCs) showing somewhat flattened morphology. Original magnifications are indicated.

PLEASE NOTE: There is no known specific marker for human corneal endothelial cells, so the ability to differentiate MSCs to corneal endothelium will be determined by a combination of morphological characteristics, Western blot studies of proteins known to be expressed by corneal endothelial cells, and, eventually, by *in vivo* opacity tests to analyze endothelial cell function.

2) Test of Effect of HCEC Culture Medium on MSC Growth Characteristics: An initial series of experiments was conducted to determine whether MSCs would grow and form a confluent monolayer by incubation in a culture medium developed in our laboratory that supports growth of HCECs[1]. This medium contains OptiMEM-1, selected nutrients, 8% fetal bovine serum (FBS), and growth factors, including epidermal growth factor (EGF) and nerve growth factor (NGF).

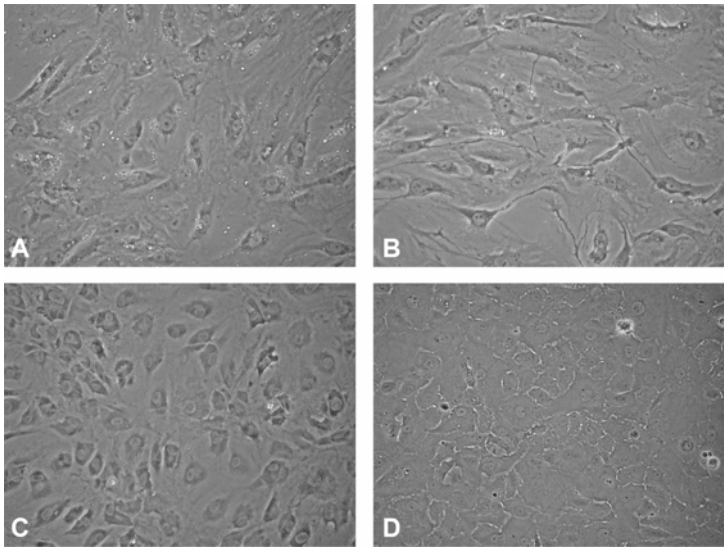


Figure 2. Effect of culture medium on MSC morphology. (A) MSCs in basal medium containing DMEM, low glucose, 10% FBS as a control. (B) MSCs cultured for 8 days in complete HCEC medium. Note the elongated cell shape and lack of cell-cell contacts. (C) MSCs cultured for 26 days in HCEC medium containing 8% FBS without additional growth factors. (D) Confluent HCECs cultured from a 75yo donor. Original magnification: 20X.

MSCs were grown for 8 days under basal conditions as a negative control (Fig. 2A) or in the medium described above that supports the growth of HCECs (Fig. 2B). Culture in this medium promoted growth of

MSCs and cells remained as a single layer; however, cell morphology changed in this medium from a somewhat rounded shape to a highly elongated, neuronal-like shape with long, sometimes branching cell processes. MSCs grown for 26 days in HCEC culture medium containing 8% FBS, but no additional growth factors, formed an apparently confluent monolayer of cells (Fig.2C). A confluent monolayer of HCECs is shown in Fig. 2D for comparison. Results indicate that MSCs are capable of growing in HCEC culture medium in both the presence and absence of additional growth factors. Growth in medium containing FBS plus additional growth factors, such as NGF, causes a significant shape change, with cells having a neuronal appearance. Growth in HCEC medium containing only 8% FBS caused cells to have a rounder morphology and to form an apparently confluent monolayer, suggesting that MSC might be able to differentiate into HCEC-like cells.

3) Western Blot Detection of Mesenchymal Neural Crest Markers. Initial studies were conducted to compare the relative expression of several marker proteins in an embryonic stem cell line (ESCs), MSCs, and HCEC cultured from young and older donors. Figure 3 provides evidence that MSCs and HCECs both express neuron-specific enolase (NSE), N-cadherin, and vimentin. As expected, expression of these mesenchymal neural crest marker proteins was generally lowest in ESCs.

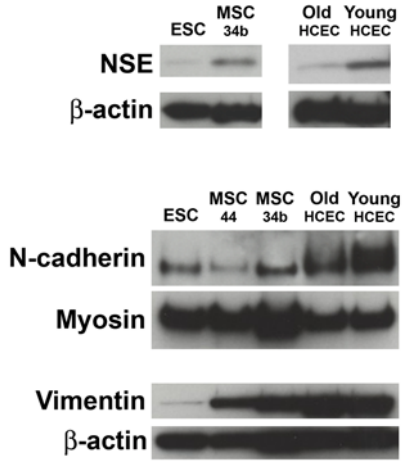


Figure 3. Western blots showing relative expression of neuron-specific enolase (NSE), N-cadherin, and vimentin in ESCs, MSCs, and HCECs cultured from young and older donors. Beta-actin or non-muscle myosin wasused a loading control based on the expected molecular weight of the test protein.

Western blots were also used to compare the relative expression of the neuronal marker, S-100, and stem cell markers, nestin, Sox2, and Musashi. Inconsistent results were obtained for these proteins, so the blots will be repeated. Overall, results indicate that MSCs express higher levels of mesenchymal neural crest marker proteins than do embryonic stem cells. MSCs and HCECs expressed similar marker proteins, showing a close relationship between these cells and providing suggestive evidence that it should be possible to find methods to differentiate MSCs into functional HCECs.

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REPORTABLE OUTCOMES: Additional studies need to be conducted prior to submitting a paper for publication. Importantly, the data obtained in these studies will be used in an R21 application to the National Eye Institute for additional funding of this project. Reportable outcomes are also needed from Zieske, Ruberti, and Gilmore.

CONCLUSIONS:

1) The morphology of MSCs can be altered depending on culture medium conditions; and 2) MSCs and HCECs share expression of a number of markers of neural crest-derived mesenchymal cells, indicating a close relationship between the two cell types. Overall, additional studies are needed to find specific conditions that will more fully differentiate MSCs to form functional HCECs; however, progress has been made toward this goal.

Subtask (4): To determine the basis for S. aureus adherance to corneal epithelial cells. (Michael Gilmore, PhD.)

Introduction:

Staphylococcus aureus is a major cause of bacterial keratitis linked with non-surgical trauma to the eye, including contact lens wear. The pathology results from a combination of bacterial toxins and the host immune response. Interestingly, some S. aureus toxins can also modulate cellular behavior at sublytic concentrations by altering receptor processing and intracellular signaling events. Most of these toxins are modulated by global regulators Agr and Sar.

The ocular surface plays an important role in innate immunity by acting as a barrier to infectious agents as well as secreting anti-microbial peptides, cytokines and chemokines following exposure to bacteria. Little is known regarding the global effects of *S. aureus* and its toxins on human corneal epithelial cells in the early phases of infections. The main goal of this study was to determine the genetic program expressed by corneal epithelial cells in response to exposure to *S. aureus* and its products at the earliest step in infection – a point where intervention might prevent subsequent pathology.

Materials and Methods:

Human corneal epithelial cells : Monolayers of Araki-Sasaki cells (hCEC) and primary human corneal epithelial cells (hCEC-3, Cascade BiologicsTM) were grown at 37ºC under 5% CO2 in defined serum-free keratinocyte media.

S. aureus strains : RN6390 (wt; toxigenic) and ALC135 (isogenic agr-/sar- mutant; non-toxigenic) were cocultured with the hCEC's

Infection Conditions : Late-logarithmic phase *S. aureus* cultures were washed and resuspended in defined SF-DKM to a multiplicity of infection (MOI) of 20 bacteria/epithelial cell. Infections were conducted at 37ºC under 5% CO2 for 6 hrs. Viability of the infected epithelial monolayer was noted to be ≥ 90%. The corresponding RN6390 culture supernatants contained hemolytic activity; whereas, ALC135 supernatants did not. Alternatively, monolayers were pretreated with cytochalasin D (0.5mg/ml) for 1hrs prior to infection or Pam3Cys (10mg/ml) only for 12hrs.

Microarray Analysis : RNA was extracted with a RNeasy Mini kit (QIAGEN), and cDNA quantification was assessed by hybridization with Affymetrix Gene Chip
Human Genome U133 Plus 2.0 Array

Cytokine Analysis : Bio-Plex Human Cytokine 8A Assay (BIO-RAD) and DuoSet ELISA human CL20/MIP-3a (R&D Systems)

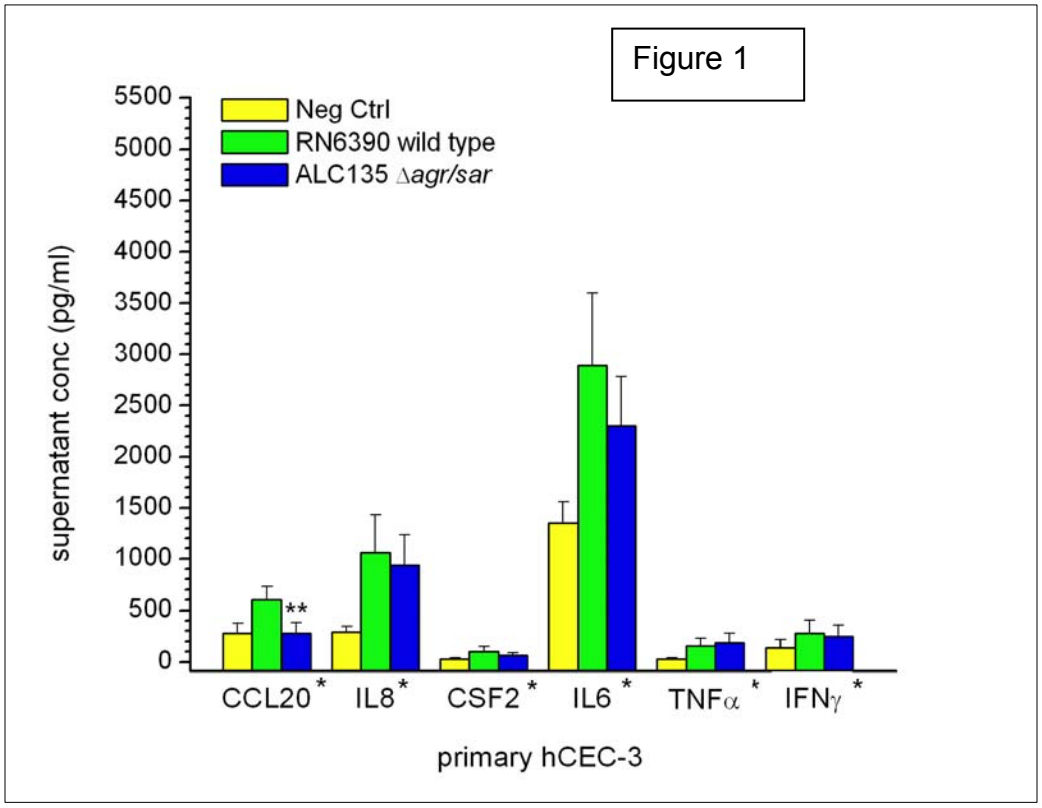
Results:

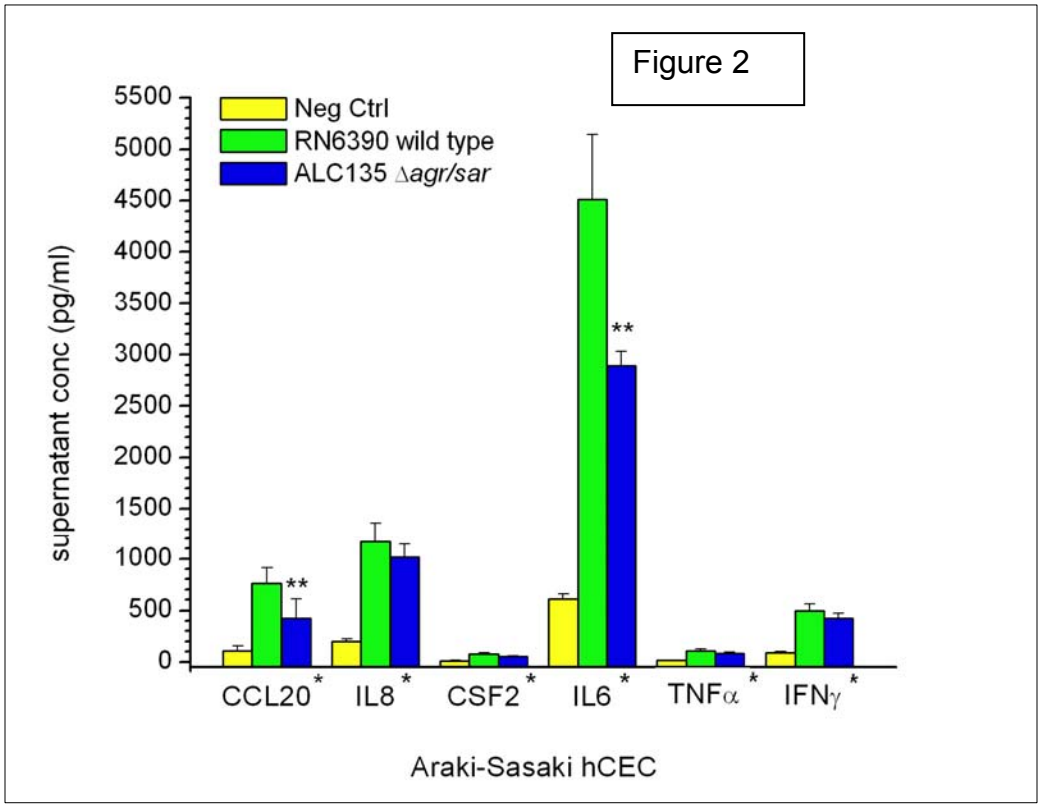
Changes in gene expression by hCEC's resulting from exposure to *S. aureus* are shown in Table 1.

Table 1.

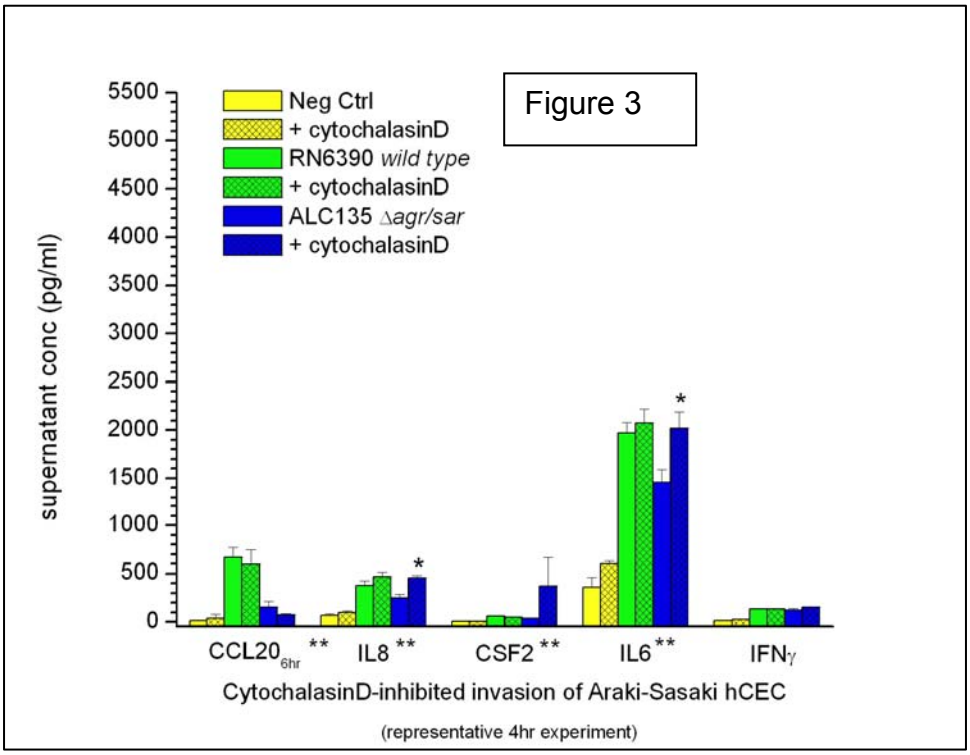
Inflammatory Molecules	**Fold Δ**	**Biological Function**
CCL20 (MIP3α)	355	*macrophage chemokine, anti-microbial.*
IL8	58	*neutrophil chemokine.*
CSF2 (GM-CSF)	42	*granulocyte/macrophage stimulator.*
CXCL1 (GRO1)	27	*neutrophil chemokine.*
IL6	19	*acute phase protein production.*
TNFα	10	*pro-inflammatory, apoptosis.*
Signaling Molecules		
Hsp70B	127	*protein folding, anti-apoptotic.*
SerpinB2	52	*monocyte transmigration, anti-apoptotic.*
TNFαAIP3	40	*negative regulator of NFκB signaling.*
FosB	40	*transcriptional regulator (AP1-Jun).*
DuSP1	34	*MAPK phosphatase.*
Inhibinβ A	31	*TGFβ family member.*
Stanniocalcin1	29	*calcium / phosphate homeostasis.*
GADD34	27	*protects from ER stress, apoptosis.*
Down-regulated Molecules		
CCNF	-6	*cell cycle regulator.*
ZAP	-5	*NAD+ ADP ribosyltransferase.*
GJA5 (Connexin 40)	-5	*gap junction assembly.*
SC4MOL	-4	*cholesterol biosynthesis.*

Changes in gene expression are reflected in secretion of proinflammatory factors into the culture fluid (Figure 1), irrespective of whether the hCEC's are primary or immortalized (Figure 2). The very high magnitude changes seen in upregulation of CCL20 are only beginning to be evident.

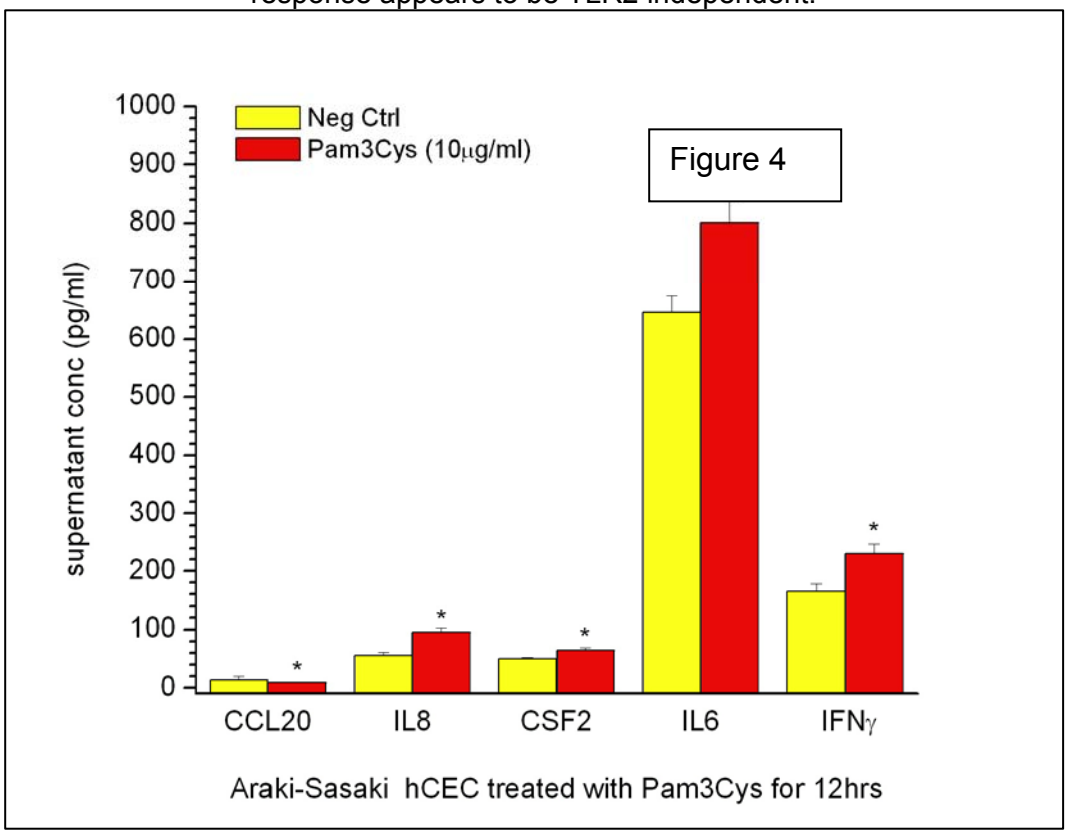




To test whether the proinflammatory response seen above related to or was influenced by the invasion of epithelial cells by *S. aureus*, a comparison was made between hCEC cultures exposed to S. aureus in the presence or absence of cytochalasin D. As shown in Figure 3, the proinflammatory response does not require hCEC invasion.



To determine whether the proinflammatory effects resulted from stimulation through toll like receptor 2 (TLR2), hCEC's were stimulated by the TLR2 inducer, Pam3Cys, and the responses compared to those generated by *S. aureus*. As shown in Figure 4, some proinflammatory effects were seen, but the CCL20 response appears to be TLR2 independent.



Conclusion:

The principle early response of hCECs to *S. aureus* is the induction of genes encoding pro-inflammatory and related-signaling molecules, including CCL20, IL8, CSF2, IL6, TNFα, and IFNγ. Changes in gene expression are only partly reflected at early time points, and additional experiments are needed to determine the ultimate impact of these changes. Bacterial invasion is not necessary to stimulate the proinflammatory response of corneal epithelium.

Task 4: Andrew Taylor, Ph.D.

Task: To regulate wound repairing macrophages within the retina of eyes with catastrophic injury.

INTRODUCTION:

The eye is an immune privileged tissue where immunity is highly regulated. This regulation is mediated by soluble factors produced within the ocular microenvironment by cells of the eye, nervous system, and immunity itself. This regulation suppresses the induction of inflammation that can cause scaring, tissue damage, and loss of vision. One of the mechanisms of suppression we discovered was that in the retina macrophages are alternatively activated to simultaneously express two enzymes nitric oxide synthase 2 (NOS2) and Arginase 1. Both are associated with suppressor type macrophages. Usually, macrophages that mediate inflammation express NOS2 and not Arginase 1 and macrophages that mediate wound repair/scar formation express Arginase 1 and not NOS2. The suppressor macrophages inhibit induction of inflammation and the activation of immunity. The suppressor macrophages are considered to have an important role in regulating immunity by preventing the induction of autoimmune disease and in diminishing the inflammation of successful immune responses. In wounded retinas, we find macrophages that mediate inflammation (expressing only NOS2) and macrophages that meditate wound repair/scar formation (expressing only Arginase 1) and no suppressor macrophages. This means that in the wounded retina the expected mechanisms regulating immunity are changed. To understand what immunoregulatory mechanisms has changed in wounded retinas, we must understand what are the mechanisms of immunoregulation in the healthy retina and see whether these mechanisms as therapy can be imposed on the wounded retina to return it to health and prevent further retinal damage from inflammation and unregulated wound repair.

BODY:

We used primary macrophages (MØ) from resident peritoneal exudate cells of C57BL/6J mice. The MØ were treated with the conditioned media (CM) of retinal pigmented epithelial cell eye cups (RPE-eyecups). These eye cups were prepared from healthy mouse eyes, and the methods for making the RPE-eyecups and for culturing the RPE-eyecups to obtain the CM was designed in a previous published DOD sponsored project.(*1*) For the experiments in the current task RPE-CM was depleted of several neuropeptides and cytokines that are suspected to have a role in retinal regulation of macrophage activity. These factors are calcitonin gene related peptide, alpha-melanocyte stimulating hormone (α-MSH), neuropeptide Y (NPY), pigment-epithelium derived factor, somatostatin, and transforming growth factor-beta. These factors have been detected in the retina, and they have important immunomodulating activities.(*2-7*) Therefore, by depleting the RPE-CM of these factors we can see if one or more of these factors are responsible for RPE-CM mediated induction of suppressor macrophages. The individual factors were depleted by treating the RPE-CM with a corresponding specific antibody. The antibodies and any bound antigen are removed from the antibody treated RPE-CM with Protein-G coated beads, the beads were spun-out, and the resulting supernatant, the depleted RPE-CM, was used in the experiments. The MØ were treated with the depleted RPE-CM, and incubated for 24 hours. The cells were formalin fixed, and stained with antibodies against the enzymes NOS2 and Arginase1. We also documented the MØ morphology, and assayed for nitric oxide (NO) production.

The MØ incubated with whole RPE-CM were morphologically activated with pseudopodia-like extensions, co-expressed Arginase 1 and NOS2, with enhanced NO production. Changes in NO production occurred only when we depleted the RPE-CM of α-MSH or NPY and not with the depletion of the other suspected cytokines, growth factors and neuropeptides. When α-MSH was absorbed, the treated MØ expressed small amounts of Arginase 1 and high levels of NOS2, and were round with a ruffled cell membrane like inactivated or dead MØ. When NPY was depleted, the treated MØ expressed only Arginase 1, and were morphologically similar to activated MØ. When both α-MSH and NPY were depleted, the treated MØ expressed small amounts of only NOS2, and were round like inactivated or dead MØ.

KEY RESEARCH ACCOMPLISHMENTS:

• Discovered that α-MSH and NPY are produced by RPE in the RPE-eyecup model.

• Discovered that α-MSH is needed for the MØ to be activated and/or survive

• Discovered that NPY mediates the expression of NOS2 in the suppressor MØ

• Further supported our hypothesis that the RPE and the retina like other parts of the eye tightly regulate immunity

REPORTABLE OUTCOMES

Abstracts to Association for Research in Vision and Ophthalmology (how many, which years?), attached in Appendix 1.

CONCLUSIONS:

The research accomplished to date shows that the RPE mediates the expression of NOS2 and Arginase1 in primary MØ through at least α-MSH and NPY. The results suggest that in the healthy retina α-MSH is an activator/survival factor of MØ with NPY and at least one more unidentified factor to make the MØ differentiate into suppressor MØ.

There are two final steps left for this task. 1) Determine if the α-MSH depleted RPE-CM are inducing cell death in the MØ. This will reveal that α-MSH acts as a master switch for MØ activation or survival in the retina. 2) Complete the task by treating the MØ with the identified factors to see if we can promote MØ differentiation into suppressor MØ, which will be the first step for future experiments to see whether it is possible to impose treatment and control the wound response in injured eyes.

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APPENDICES:

Appendix I:

Molecular characterization of retinal pigmented epithelial (RPE) mediated induction of suppressor macrophages

N. Kawanaka and A. W. Taylor

Schepens Eye Research Institute & Department of Ophthalmology, Harvard Medical School, Boston, MA, USA

Purpose:

We previously demonstrated that healthy retinas have suppressor macrophages (MØ) detected by the simultaneous expression of nitric oxide synthase 2 (NOS2) and Arginase 1. In addition, we found that in wounded retinas the MØ are either inflammatory MØ (only expressing NOS2), or wound repairing MØ (only expressing Arginase1). To understand how this change can happen, we need to identify the soluble factors produced by RPE cells responsible for regulating macrophage functionality in the healthy retina.

Methods:

Our source of primary MØ were from resident peritoneal exudate cells of C57BL/6J mice. The MØ were treated with the conditioned media (CM) of RPE-eyecups as described previously; however, this time the RPE-CM were absorbed of several suspected neuropeptides and cytokines (CGRP, a-MSH, NPY, PEDF, SOM, and TGF-□) using their corresponding specific antibodies and Protein-G coated beads. The treated MØ were incubated for 24 hours, formalin fixed, and stained with antibodies against the enzymes NOS2 and Arginase1. We also documented the MØ morphology, and assayed nitric oxide (NO) production by the MØ.

Results:

The MØ incubated with CM were morphologically activated, co-expressed Arginase 1 and NOS2, with enhanced NO production. Changes in enhanced NO production occurred only when we absorbed the CM of □-MSH or NPY. When □-MSH was absorbed, the CM treated MØ expressed only small amounts of Arginase 1 and NOS2, and were round like inactivated MØ. When NPY was absorbed, the treated MØ expressed only Arginase 1, and were morphologically similar to activated MØ. When both were absorbed, the treated MØ expressed only small amounts of NOS2, and were round like inactivated MØ.

Conclusion:

These findings show that RPE mediates the expression of NOS2 and Arginase in primary MØ through at least □-MSH and NPY. The results suggest that in the healthy retina □-MSH is an activator of MØ, and with NPY makes the MØ differentiate into suppressor MØ.

Supported in part by PHS grant EY10752, and the DOD W81XWH-04-1-0892

TASK 5: Reza Dana

Task: To modulate corneal inflammation through chemokine receptor regulation.

INTRODUCTION:

The purpose of this project is to determine the functional relevance of chemokines (small molecular weight cytokines that provide directional information to leukocytes, and are critical for immunocyte activation and homing and chemokine receptors that mediate corneal immunity and inflammation. Corneal inflammation, as a result of trauma, is one of the leading morbidities suffered by active military personnel, and is a leading cause of visual impairment and blindness. The current treatments for corneal inflammation are limited, both due to limited efficacy in preventing/suppressing destructive inflammation as well as due to myriad side-effects, such as induction of glaucoma and cataracts by corticosteroids. Therefore, determining novel targets for controlling inflammation is a priority in the field of ocular inflammation.

BODY:

The Statement of Work for our project was "modulation of corneal inflammation through chemokine receptor regulation". To accomplish this goal, we made use of an animal model of corneal orthotopic allografting. This model has high value for several reasons. First, corneal transplantation remains the principal strategy for treating large/significant corneal scars suffered from injuries. Second, it faithfully creates the clinical condition of corneal transplantation in the laboratory. Third, it has been used extensively as a model to study the molecular mechanisms of inflammation in the eye. Fourth, from an immunological standpoint, this model has also considerable value since it sets a "high bar" for demonstrating efficacy in immunomodulatory strategies.

In the first set of experiments, corneal transplants were performed amongst fully mismatched strains. Cytokine and chemokine receptor gene expression was determined by the ribonuclease protection assay. Knockout (KO) strains for chemokines/chemokine receptors that were upregulated after transplantation underwent transplantation. Results derived from KO murine strains were compared with cyclosporine-A (CsA) therapy. We determined that chemokine receptors CCR1, CCR2, and CCR5 were upregulated after transplantation. However, among the various ligand and receptor KOs tested, only CCR1 KOs showed enhanced survival, even compared to systemic CsA therapy. This was associated with suppressed corneal infiltration and cytokine expression.

In the second set of experiments, the trafficking of corneal antigen-presenting cells (APCs) to secondary lymphoid tissues (draining lymph nodes) was studied. Specifically, the expression and function of the chemokine receptor CCR7 was evaluated in mediating APC mobilization in corneal inflammation. Expression of CCR7 and its ligands CCL19 and CCL21 were analyzed by RT-PCR in normal and inflamed corneas. Alexa488-conjugated ovalbumin was injected into corneal transplants to use as a marker for trafficking of graft-borne APCs to the draining lymph nodes. We determined that both CCR7 and its ligand CCL21 are significantly upregulated in inflamed corneas. The CCR7+ cells were CD11b+CD11c+ and a majority were MHC class II+ suggesting a monocytic dendritic lineage. Importantly, blockade of CCL21 with antibody led to a significant suppression in the flow of OVA+CD11c+ cells to the draining lymph nodes.

KEY RESEARCH ACCOMPLISHMENTS:

- Evidence, for the first time, that targeting of specific chemokine pathways can significantly promote survival of corneal transplants and associated corneal inflammation
- Identification of a novel chemokine system which enables antigen-bearing APCs to get mobilized from cornea into lymphoid tissues, enabling induction of T cell-mediated immunity

REPORTABLE OUTCOMES:

- Published two peer-reviewed articles summarizing above data (include in appendix?)
- Identified novel pathways for regulation of corneal inflammation and immunity which may also serve as therapeutic targets

CONCLUSIONS:

The World Health Organization (WHO) has identified corneal inflammation, and resultant scarring and neovascularization, as the second most common causes of blindness worldwide (after cataracts). Among the military, far in the way the #1 cause of ocular morbidity amongst the active personnel is corneal and ocular surface disease and trauma. While for minor injuries corticosteroids are effective if used for a limited period, they have limited efficacy in many cases of severe injury. Additionally, their use is associated with secondary infections, glaucoma, and cataracts. Therefore, development of new therapeutic targets is a major goal for providing better care to these patients. The studies afforded by this funding mechanism have allowed for identification of new chemokine-mediated mechanisms that promote infammation; as such, these receptor-ligand systems may serve as new targets for immune modulation in ocular inflammation.

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2. Jin Y, Shen L, Chong EM, Hamrah P, Zhang Q, Chen L, Dana MR. The chemokine receptor CCR7 mediates corneal antigen-presenting cell trafficking. *Mol Vision* 2007; 13: 626-634.

TASK 6: Meredith Gregory-Ksander, Ph.D., & Michael S. Gilmore, Ph.D.

Task: To identify the innate immune effector mechanisms which determine resistance versus susceptibility to destructive *S. aureus* endophthalmitis.

Introduction:

Endophthalmitis is an infection of the posterior segment of the eye that occurs as a complication of either globe-penetrating injury, or ocular surgery and often results in significant retinal damage and loss of vision. Bacterial clearance from the eye is dependent upon a dynamic and complex host immune response characterized by an early recruitment and infiltration of neutrophils that play a critical role in clearing the bacteria (1). Unfortunately, the same factors produced by the neutrophils during bacterial clearance can also damage the local retinal tissue resulting in loss of vision. We predict that the maximum amount of ocular tissue is destroyed when a "smoldering" innate immune response slowly clears the infection. This occurs when non-specific immune effector cells destroy normal uninfected cells that are close to the site of infection. A slow response also increases the risk that the infection will not be contained and will spread throughout the eye. Bacteria possess a variety of mechanisms used to avoid and/or thwart innate immune effector cells. When an infection is not cleared quickly, there is an increasing risk that the bacteria will successfully deploy these defenses.

The studies purposed herein will identify the key innate effector mechanisms central to resistance to *S. aureus* endophthalmitis and will serve as the basis for the development of new therapies that specifically manipulate innate effector cells to increase resistance to destructive endophthalmitis.

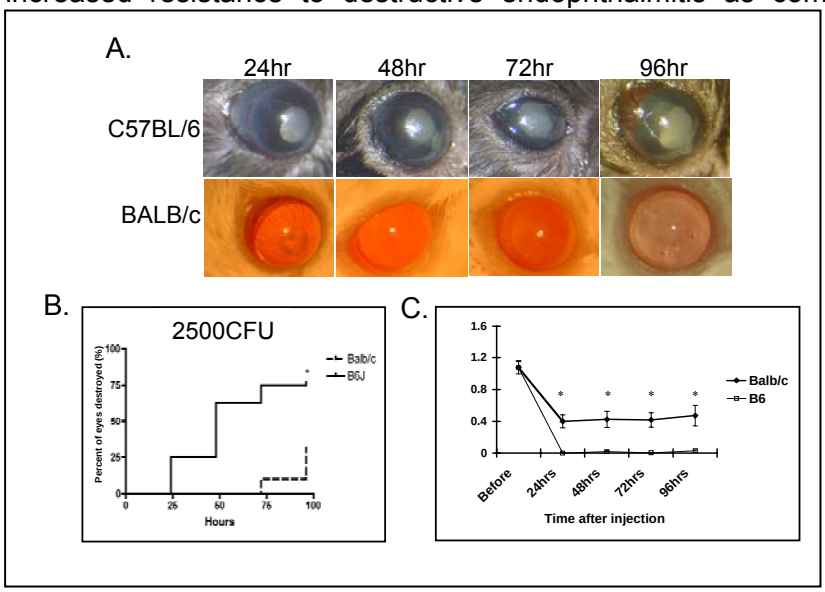
Progress Report:

Subtask 1: Quantify the immune cell influx in resistant (BALB/c) versus susceptible (C57BL/6) mice. Our laboratory has identified two genetically inbred strains of mice that display dramatically different levels of resistance to *S. aureus* induced endophthalmitis. C57BL/6 mice are highly susceptible to destructive endophthalmitis, while BALB/c mice display increased resistance and clear an identical posterior challenge of *S. aureus*. We utilized a mouse model of *S. aureus* endophthalmitis to better understand what determines susceptibility to destructive endophthalmitis. C57BL/6 and BALB/c mice received an intravitreal injection of 2500 CFU *S. aureus*. Clinical examinations, and bacterial quantification were performed at 24, 48, 72, and 96 hours post injection. Retinal damage was assessed by electroretinography and histology. The myeloperoxaidse (MPO) assay was used to quantitate neutrophil infiltration.

C57BL/6 mice infected with 2500 CFU *S. aureus* developed a progressive infection that resulted in complete destruction of the eye in 80% of the mice, as determined by clinical exams and electroretinogram (ERG) (Figure 1). By contrast, BALB/c mice displayed increased resistance to infection and complete destruction occurred in only 25% of the mice. In addition, in mice that successfully cleared the infection, (75% of BALB/c and 20% of C57BL/6) BALB/c mice displayed decreased retinal damage and increased retinal function (data not shown). Bacterial quantification at 48 hours revealed a delay in bacterial clearance in C57BL/6 mice (3×10^7 CFU/eye) as compared to BALB/c mice (1×10^5 CFU/eye) (Figure 2). This corresponded with a decreased neutrophil infiltrate in C57BL/6 mice as compared to BALB/c mice (Figure 3). These data reveal a quicker more robust neutrophil infiltration in resistant BALB/c mice as compared to susceptible C57BL/6 mice. Furthermore, the early neutrophil infiltrate corresponds with a more efficient clearance of *S. aureus,* preventing destructive endophthalmitis.

Additional time points (48hr, 72hr, 96hr) are currently being examined to determine whether a delayed and prolonged neutrophil infiltrate corresponds with the increased retinal damage observed in the susceptible C57BL/6 mice. We propose that a slow "smoldering" innate immune response is ineffective at clearing the *S.aureus* and will cause increased non-specific host tissue damage.

Figure 1: BALB/c mice display increased resistance to destructive endophthalmitis as compared to C57BL/6 mice. BALB/C and C57BL/6 mice received an intravitreal inoculation of 2,500 CFU S.aureus. At 24, 48, 72 and 96 hours following injection, clinical examinations were performed by slit lamp biomicroscopy and each eye was given a score of 0, 1, 2, or 3, (0=cleared and 3=destroyed). At 96 hours complete destruction was observed in 80% of C57BL/6 mice, while destruction only occurred in 25% of BALB/c mice (A, B). Electroretinography (ERG) revealed a complete loss of retinal function in C57BL/6 mice as compared to BALB/c mice (C).



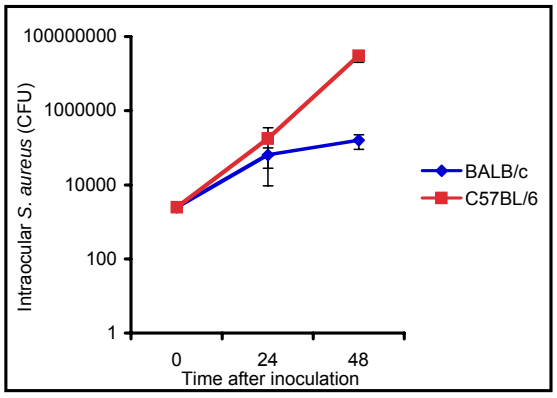


Figure 2: C57BL/6 mice display an increased bacterial load at 48 hours post inoculation as compared to BALB/c mice. BALB/c and C57BL/6 mice received an intravitreal inoculation of 2,500 CFU S. aureus. Mice were euthanized at 48 hours and eyes were enucleated and processed for bacterial quantification. The eyes of C57BL/6 mice contained 3 x 10^7 CFU/eye as compared to 1 x 10^5 CFU/eye in BALB/c mice. THIS FIGURE MOVED!

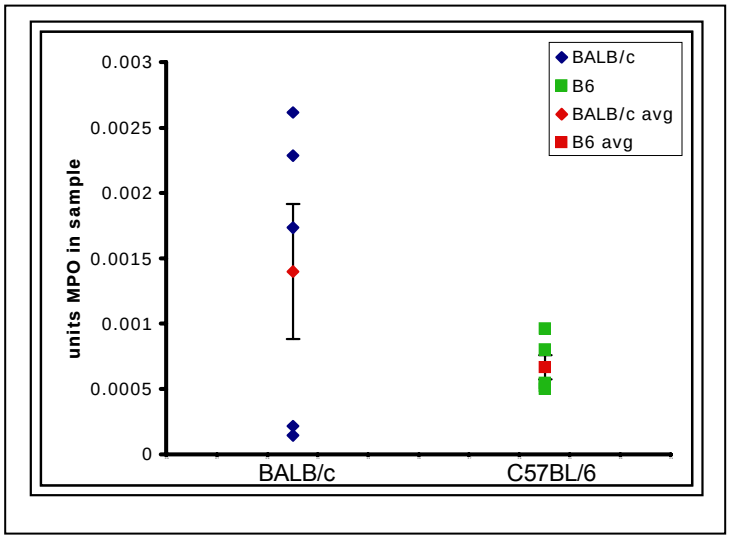


Figure 3: C57BL/6 mice display a delayed recruitment of neutrophils at 24 hours. BALB/c and C57BL/6 mice received an intravitreal inoculation of 2,500 CFU S. aureus. Mice were euthanized at 48 hours and eyes were enucleated and processed for neutrophil quantification using the myeloperoxidase assay (MPO). The MPO assay revealed a more rapid and robust neutrophil infiltrate in BALB/c mice at 24 hours post inoculation as compared to C57BL/6 mice.

Subtask 2: Identify the cytokines produced and examine the kinetics of production as they relate to the course of disease. Experiments are currently underway using Luminex to examine the cytokines produced in the posterior segment of resistant BALB/c versus susceptible C57BL/6 mice.

Subtask 3: Assess activation and phagocytic properties of infiltrating macrophages and neutrophils. Experiments are currently underway

Key Research Accomplishments:

(1) We identified resistant (BALB/c) and susceptible (C57BL/6) strains of mice that we can now use to identify the innate effector cell mechanisms required for resistance to destructive *S. aureus* endophthalmitis.

(2) We demonstrated that resistance to destructive endophthalmitis corresponds to a more rapid and robust neutrophil infiltrate. A delayed neutrophil infiltration corresponds with destructive endophthalmitis. These results demonstrate the importance of the neutrophil mediated response in bacterial clearance.

Reportable Outcomes:

(1) Abstract submitted to ARVO 2008:

> C. Hackett, E Whiston, N Sugi, BR Ksander, MS Gilmore, and MS Gregory. Resistance to infection and protection of retinal tissue during S. aureus endophthalmitis

(2) Paper in preparation. Expected to be submitted in Spring 2008

Conclusions:

Our data demonstrate that increased resistance to destructive endophthalmitis corresponds with a rapid and robust neutrophil infiltration. These results demonstrate the importance of the neutrophil mediated response in bacterial clearance and bring attention to the difficult question of when or if anti-inflammatory medication should be administered to patients suffering form endophthalmitis. Additional experiments are currently being performed to determine why susceptible C57BL/6 mice present with delayed neutrophil infiltration and poor bacterial clearance. These studies will examine cytokine and chemokine production (Subtask 2) and neutrophil activation and phagoytosis (Subtask 3).

References:

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TASK 7: Andrius Kazlauskas, Ph.D.

Task: To determine the mechanism by which priming licenses cells to engage the cell cycle.

INTRODUCTION:

Control of cell proliferation is highly relevant to military personnel both on and off the battlefield. Non-lethal encounters with improvised explosive devices result in soldiers needing repair and regeneration of tissue surrounding the eyes. Similarly, wound repair within the eye for soldiers off the battlefield are important for flap stability in LASIK patients, and control of haze following PRK. Understanding how cell proliferation is regulated will dramatically improve our ability to modulate wound repair and thereby address a key military need.

The traditional view of how growth factors advance quiescent cells into the cell cycle is that cells first encounter a competence factor, which makes them capable of exiting G0. Subsequent exposure to a second type of growth factor (a progression factor) drives cells out of G0, through G1 and past the restriction point, whereupon cells are committed to one round of the cell cycle (*1*).

Since the introduction of these concepts approximately 35 years ago, the major advances in this field have been to molecularly define the components of the cell cycle program and the restriction point (*2-4*). We now know that the restriction point consists of inactivation of Rb, which results from its phosphorylation by cyclin dependent kinases that are activated in response to a growth factor-driven rise in cyclins and fall of cyclin dependent kinase inhibitors during the second half of G1. We also know the biochemical details of the signaling events that are triggered by exposure of quiescent cells to growth factors. What we do not know is how these early signaling events license cells to engage the cell cycle program.

Our research in the last 5 years has provided the conceptual framework necessary to investigate how growth factors license cells to enter the cell cycle (*5*). We discovered that the G1 phase of the cell cycle consists of two distinct growth factor-dependent segments. The first is a priming phase, during which cells prepare to engage the cell cycle program, and the second segment is a completion phase, during which the cell cycle program is active (*6, 7*).

Our *working hypothesis* is that the initial exposure of cells to growth factors primes them by inducing the expression of priming proteins. Subsequent exposure to growth factors results in the tyrosine phosphorylation of the priming proteins and thereby licenses cells to engage the cell cycle program.

The specific objective of this proposal is to molecularly define the priming phase of G1. The resulting information will reveal how growth factors license cells to enter the cell cycle. These scientific advances will constitute the foundation for developing effective approaches to promote would healing in tissue within and immediately surrounding the eye.

BODY:

We have focused on optimizing the priming protocol. While this approach was developed in my lab, it is a difficult assay that has considerable variability between different investigators. Consequently, we sought to optimize the protocol so that it would work well and reproducibly for the vast majority of investigators that employed it. We tried a variety of formulation for the buffer to strip platelet-derived growth factor (PDGF) off of the cells. In addition, we attempted to replace PDGF with growth factors that adhere less tightly to cells and hence are easier to remove. We have not been able to further optimize the original protocol (*7*), and concluded that varying some of the most obvious parameters did not improve upon the assay.

KEY RESEARCH ACCOMPLISHMENTS:

- Varying a series of key parameters did not further optimize the priming protocol.

REPORTABLE OUTCOMES:

Kazlauskas A. The priming/completion paradigm to explain growth factor-dependent cell cycle progression. Growth Factors. 2005; 23:203-10.

CONCLUSIONS:

The current protocol is optimized for priming cells.

REFERENCES:

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TASK 8: Michael Young, Ph.D.

Task: To develop novel biopolymer constructs fro treatment of retinal diseases and injury by seeding polymer scaffolds will be seeded with retinal progenitor cells, which can in turn be combined with retinal tissue in culture to generate a composite retinal graft. This graft will be juxtaposed to degenerating retina in vivo and in vitro.

INTRODUCTION:

There is no restorative treatment available for retinal tissue destroyed by degenrative diseases like Retinitus Pigmentosa and Age-Related Macular Degeneration. Tissue engineering using biodegradable polymers seeded with retinal progenitor cells (RPCs) offers new hope as a potential cell replacement therapy to restore lost retinal tissue.

BODY:

Our current tissue engineering objectives are to develop new strategies to direct retinal progenitor cells (RPCs) toward a photoreceptor fate on biodegradable polymers, to be transplanted for the anatomic and functional restoration of degenerated retina. In collaboration with the bioengineering laboratories of Robert Langer at the Massachusettes Institute of Technology, Gary Wnek at Case Western Reserve University, and Tejal Desai at University of California, San Francisco, the co-development of smart polymers that facilitate retinal progenitor cell survival following subretinal transplantation is a primary focus.

We have co-designed ultra-thin (~6um) poly(methyl methacrylate) (PMMA) scaffolds (3) The new PMMA scaffolds were micromachined to contain (~20μm) through pores for the (Eliminate the paragraph space)

retention of RPCs. Porous PMMA was shown to provide significantly greater adhesion for RPCs when compared to un-machined (non-porous) versions. The ultra thin PMMA-RPC composites were then transplanted into the sub-retinal space of C57bL/6 mice, caused no observable damage to surrounding issue, and allowed for the migration of cells into each layer of the host retina by two weeks in vivo. Currently, both microfabricated poly(glycerol sebacate) (PGS) (2) and template synthesized poly(capro lactone) (PCL) (1) polymer scaffolds are being evaluated for rtinal tissue engineering capabilities.

Porous PGS scaffold with a thickness of 45 μm was fabricated suing a microfabrication process. Analysis of the elastic properties of this scaffold showed that the Young's modulus is about 5-fold lower and the maximum elongation at failure is about 10-fold higher than the previously reported RPC scaffolds. The elasticity of porous PGS allows the scaffold to be coated with RPCs, scrolled into a needle tip and injected into the sub-retinal space (2). RPCs strongly adhered to the poly(glycerol-sebacate) scaffold, and endogenous fluorescence nearly doubled over a 2-day period before leveling off after 3 days. Immunohistochemistry revealed that cells grown on the scaffold for 7 days expressed a mixture of immature and mature markers, suggesting a tendency towards differentiation. We observed that, microfabricated poly(glycerol-sebacate) exhibits a number of novel properties for use as a scaffold for RPC delivery.

RPCs were cultured on smooth, short nanowire (2.5μm), and long nanowire (27μm) PCL scaffolds, produced using a template synthesis technique. RPC survival and proliferation is enhanced on nanowire PCL scaffolds when compared to smooth controls. Immunohistochemistry revealed that both short and long nanowire PCL scaffold topology influenced protein expression levels in RPCs. Mouse RPCs cultured on nanowire scaffolds demonstrated differentiation including increased expression of the bipolar cell marker PKC and the photoreceptor markers recoverin and rhodopsin. RT-PCR revealed the down regulation of early, unidifferentiated, RPC genes and and up-regulation of the glial cell marker GFAP. Scaffolds seeded with RPCs and transplanted onto C57bl/6 and rhodopsin knockout mice retinal explants in vitro or in vivo into the sub-retinal space allowed for migration and differentiation of RPCs to each retinal layer

KEY RESEARCH ACCOMPLISHMENTS:

Stem and progenitor cells can be combined with template synthesized and microfabricated polymer substrates for the generation of tissue equivalents in culture.

RPCs delivered using these polymers migrate into the host retina, extend processes, and undergo differentiation.

These micro-engineered polymer scaffolds provide a biomimetic environment for tissue engineering and transplantation into the diseased eye.

REPORTABLE OUTCOMES:

1. Redenti S., Tao S., Zhang Y., Klassen H., Desai T. and Young. 2008. Tissue Engineering using Mouse Retinal Progenitor Cells and a Novel Biodegradable Poly(e-caprolactone) Nanofiber Scaffold. ARVO Abstract
2. Neeley WL, Redenti S, Klassen H, Tao S, Desai T, Young MJ, Langer R. A microfabricated scaffold for retinal progenitor cell grafting. Biomaterials. 2008. Feb;29(4):418-26. Epub 2007 Oct 24
3. Tao S., Young C., Redenti S., Zhang Y., Klassen H., Desai T. and Young MJ. 2007. Survival, migration and differentiation of retinal progenitor cells transplanted on micro-machined poly (methyl methacrylate) scaffolds to the subretinal space. Lab on a Chip. Jun;7(6):695-701.

CONCLUSIONS:

The results suggest that the PCL-RPC composites allow for precise delivery of high numbers of RPCs differentiated towards specific retinal neural fates for the potential replacement and restoration of retinal tissue destroyed by disease or trauma.